

INTRAUTERINE GROWTH RESTRICTION ALTERS
HYPOTHALAMIC PROGRAMMING
IN THE DEVELOPING
MOUSE BRAIN

by

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ABSTRACT

Intrauterine growth restriction (IUGR) creates altered programming in many organs, including the hypothalamus. Programming involves adaptive changes initiated to protect the survival of a fetus following IUGR, which can adversely influence the later response of genes that regulating energy balance. In male mice, IUGR coupled with rapid postnatal catch-up growth predisposes towards early onset obesity. One potential mechanism for this obesity is altered hypothalamic programming of intrahypothalamic cues involving prepro-orexin and prepro-MCH, two energy balance regulating genes as well as the extra-hypothalamic cues of peripheral hormones leptin and insulin. We therefore hypothesized that IUGR would alter hypothalamic programming of prepro-orexin and prepro-MCH genes in association with altered serum leptin and insulin levels in male mice at postnatal day 7 (P7) and P60.

IUGR was induced via maternal thromboxane A₂-analog infusion in the last week of C57BL/6J mouse gestation. Sham operated dams acted as controls. Sham and IUGR offspring were cross-fostered to unmanipulated dams and weaned to standard mouse chow at P21. In this mouse model, IUGR males, as compared to IUGR females, achieve rapid catch-up growth to sham males by P28 whereas IUGR females do not catch-up to sham females until P77. We measured mRNA levels via quantitative real-time RT-PCR, protein levels via Western immunoblotting, serum leptin and insulin levels via ELISA, and food intake from P21 to P60.

IUGR increased hypothalamic prepro-orexin protein levels and decreased MCH mRNA levels in P7 IUGR males. In conclusion, our findings of IUGR-induced altered hypothalamic programming of prepro-orexin and prepro-MCH are gender-specific and do not persist in the neonatal period, but into adulthood. Altered programming of genes such as prepro-orexin and prepro-MCH in the neonatal period may contribute to the differential catch-up growth observed between IUGR male and female mice in this model.

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I. INTRODUCTION

Intrauterine Growth Restriction

The obesity epidemic is escalating disproportionately in the United States for adults, and now for children and adolescents (1,2). One identified predisposing factor for early onset obesity among children and adolescents is intrauterine growth restriction (IUGR) (3). IUGR occurs when a decrease in transplacental transfer of oxygen and nutrients occurs in the fetus (5). The altered prenatal environment causes subsequent changes in gene expression to ensure survival of the fetus (6). These adaptive changes, however, have been shown to impact metabolic processes that lead to an increased risk of postnatal obesity, as well as other co-morbidities such as type 2 diabetes, cardiovascular disease, and insulin resistance (3,4,6). Furthermore, IUGR coupled with rapid postnatal catch-up growth, strongly predicts an earlier onset of obesity (16). The connection between IUGR and obesity is well-demonstrated in human and animal models of IUGR (7).

The concept that a prenatal insult, such as IUGR, can manifest adult onset disease, such as obesity, is referred to as programming, and is a phenomenon that is well described in animals and humans. Programming generally refers to adaptive changes made to ensure survival in the perinatal environment profoundly impacting the final phenotype throughout life. These adaptive changes can adversely impact many organs, including the brain (3,8). Within the brain, the hypothalamus has an established role in

controlling energy balance (3). Therefore, any IUGR- induced programming that may occur within the hypothalamus will alter the metabolic fate of the IUGR infant, child and adult.

Hypothalamus

The hypothalamic neurons and axons that aid in energy balance regulation develop during perinatal life in rodents (3). During this critical time of development, external and internal cues influence hypothalamic programming and influence the final phenotype. Intra-hypothalamic cues also help to organize the hypothalamus. These cues refer to the actual neurons that make up the arcuate nucleus ARC of the hypothalamus. The ARC is anatomically posed to act as the main hub in the hypothalamus regarding energy balance (10). The area of interest for this study is the lateral hypothalamic area (LHA) which receive projections from the ARC. The LHA contains two populations of neurons that produce orexigenic neuropeptides which impact energy homeostasis in humans and rodents (13). One group produces orexin, while the other produces melanin concentrating hormone (MCH) in both humans and rodents (3,11,12).

Extra-hypothalamic cues include hormones released from peripheral organs such as leptin produced by adipocytes and insulin produced by the pancreas. Both leptin and insulin are integral in creating a normally functioning hypothalamus in animals and humans (3). An appropriate leptin surge at the correct time is essential in the early neonatal environment to solidify hypothalamic circuits; thereby ensuring appropriate signaling or programming for normal energy homeostasis in rodents (3, 9,17,19). Additionally, if increased levels of insulin occur during hypothalamic development,

obesity and unfavorable neuronal morphology to the hypothalamic ARC is evident in rodents (3,17).

Orexin and Melanin Concentrating Hormone

Orexin, also known as hypocretin, encompasses both orexin A and orexin B neuropeptides. Both neuropeptides are produced from a common precursor polypeptide named prepro-orexin (12). Orexin A and B bind to two specific-G-protein-coupled receptors, OX1R and OX2R for downstream signaling (12,13). Similarly, MCH is produced from an MCH precursor and interacts with two specific-G-protein-coupled receptors, MCH1R and MCH2R (11,14,20). For the purpose of this project, we will only examine the levels of hypothalamic prepro-orexin and prepro-MCH, acknowledging that both receptors are present throughout the brain and are required for cellular action (12). Neurons that contain orexin in the LHA in humans and rodents have been shown to be sensitive to changes in nutrient levels; in fact, while in the fed state hyperpolarization and cessation of action potentials occurs in orexin neurons to decrease signal for nutrients (12). Furthermore, in studies involving prepro-orexin knockout mice, they ate less and had a decrease in energy expenditure leading to weight gain when compared to control mice, demonstrating prepro-orexin's role in energy balance (13). Similarly, MCH knockout mice showed evidence of resistance to diet-induced obesity as well as increased energy expenditure and locomotor activity as compared to control mice (10,15).

IUGR Mouse Model

Animal models are useful tools in dissecting mechanism of all diseases, including obesity. One such animal model showed that IUGR rats developed obesity following a

rapid, postnatal catch-up growth at the beginning of life (19). In our lab, we have also demonstrated such catch-up growth in a newly developed mouse model. In this mouse model, IUGR males, who are 15 percent lighter than controls at birth, achieve rapid catch-up growth to that of sham males by postnatal day 28 (P28) whereas IUGR females do not catch-up to sham females until P77. Additionally, IUGR males begin to exhibit overt obesity - as they surpass sham males in weight at P238 (8 1/2 months) whereas IUGR females do not surpass sham females in weight up to one year of age (8).

Hypothesis

We hypothesized that IUGR would alter hypothalamic programming of prepro-orexin and prepro-MCH genes in association with altered serum leptin and insulin levels in IUGR male mice at P7 and P60.

II. METHODOLOGY/RESEARCH DESIGN

Animal Model

The model for this project is a mouse model of chronic uteroplacental insufficiency achieved via micro-osmotic pump infusion of thromboxane A₂ during the last week of C57BL/6J mouse gestation (8). The resultant maternal phenotype mimics complications of human preeclampsia, which is a condition of pregnancy-induced hypertension. When compared to sham-operated pups, IUGR pups show an altered metabolic profile in the blood, and display altered hepatic gene expression in genes important for growth and metabolism.

C57BL/6J male and female mice were mated. At embryonic day 12.5, pregnant females were anesthetized for micro-osmotic pump insertion (model 1007D, 0.5µl/hr, Durect Corporation, Cupertino, CA). Pumps in sham-operated mothers were filled with vehicle (0.5% ethanol) whereas pumps in IUGR mothers were filled with U-46619, a thromboxane A₂ (TXA₂) analog, dissolved in 0.5% ethanol. Pups were allowed to deliver spontaneously and cross-fostered to unmanipulated dams until weaning. Pups were separated into the following groups: IUGR males (IM), sham males (SM), IUGR females (IF), and sham females (SF). Each group included 6-8 pups, with 1-2 pups derived from different litters, in order to maximize litter-to-litter variation. Blood and hypothalami were collected at P7 and P60 for analyses.

RNA Isolation, cDNA Synthesis, RT-PCR for
Orexin and MCH mRNA Levels

Genomic-free total RNA was isolated from P7 and P60 hypothalami of sham and TXA₂ -induced IUGR mice using NucleoSpin RNA XS protocol and NucleoSpin RNA II protocol (Clontech Laboratories, Mountain View, CA) respectively. Total RNA concentration was quantified with Biotek Epoch spectrophotometer (Biotek instruments, Winooski, Vt); RNA integrity was then verified by RNA denaturing gel electrophoresis.

cDNA was synthesized for 4 ug of total RNA using random hexamers from a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Using real-time RT-PCR, P7 and P60 prepro-orexin and prepro-MCH mRNA levels were determined. All primers and probes for real-time RT-PCR were available from Taqman Assays-on-Demand with assay identification numbers of rn00565995_m1 (orexin) and mm01242886_g1 (MCH). Dye emission was detected using an automated sequence detector, ABI Prism 7900HT Sequence Detection System (SDS), in conjunction with its software version 2.1 (Applied Biosystems, Foster City, CA). Each Sample was run in quadruplicates. Relative quantitation comparing $2^{-\Delta C_t}$ (Applied Biosystems, Foster City, CA) values were used to analyze changes in gene expression between sham and IUGR pups with GAPDH as a loading control. To validate GAPDH as an appropriate loading control, parallel serial dilutions between sham and IUGR cDNA were quantified as described previously. We also ensured that the amplification efficiencies between the target genes and GAPDH were comparable.

Prepro-orexin and Prepro-MCH Protein Levels

Hypothalami obtained from P7 and P60 sham and TXA₂ mice were lysed in RIPA buffer to prepare total protein fractions for western immunoblotting. Total protein concentration was determined via BCA Protein Assay (Pierce Biotechnology, Rockford, IL) using bovine serum albumin (BSA) as standards. Protein and molecular weight markers were loaded for immunoblotting as follows: 10 uL molecular weight marker, P7 prepro-orexin females and males: 100 ug, 30 ug protein; P60 prepro-orexin females and males: 30 ug protein; P7 prepro-MCH females and males: 150 ug protein; and P60 prepro-MCH females and males: 150 ug, 100 ug protein. Proteins were separated by gel electrophoresis with 16.5% Tris-Tricine/Peptide 1.0 mm Criterion gels (Bio-Rad Laboratories, Hercules, CA) at 150V for 30 minutes. After electrophoresis, protein was transferred to polyvinylidene fluoride membranes for P60 orexin (Millipore Corporation, Billerica, MA) and nitro-cellulose membranes (Pall Life Sciences, Ann Harbor, MI) for the remaining proteins, for 30 minutes on ice. Post-transfer, membranes were blocked in 5% milk/Tris-buffered saline Tween (TBS-T) at RT for 1-1.5 h and washed in 1X TBS-T after blocking is complete. Bound proteins were then detected with antibodies against orexin (Millipore, Temecula, CA) and MCH (Millipore, Temecula, CA). GAPDH (Cell Signaling Technology, Danvers, MA) served as the loading control. Blots were incubated with each primary antibody at 4 C overnight. After multiple washes in TBS-T, membranes were probed with secondary antibody for 1 h at RT. After washing thoroughly with TBS-T, antibody signals were detected by Western Lighting enhanced chemiluminescence (PerkinElmer, Waltham, MA) and quantified with Kodak Image Station 200R (Eastman Kodak/SIS, Rochester, NY).

Leptin

Serum leptin levels were measured at P7 and P60 using RayBio Mouse Leptin ELISA kit (ELM-Leptin-001, RayBiotech, Inc., Norcross, GA). Using the quantitative sandwich enzyme immunoassay technique, standards with 3.6 uL P7 serum for 1:100 dilution and 2 uL P60 serum for 1:250 dilution were pipetted into wells that were coated with polyclonal antibody specific for mouse leptin. Any serum mouse leptin would be bound by the antibody within the wells. Next, enzyme-linked secondary polyclonal antibody for mouse leptin were pipetted into the wells. A substrate solution was then added to create a colorimetric reaction that could be measured by a spectrophotometer at 450 nm. Absorbance values of known leptin standards were then plotted against leptin concentration on a linear-regression standard curve.

Insulin

Serum insulin levels were measured at P7 and P60 using Mercodia Ultrasensitive Mouse Insulin ELISA kit (10-1249-01, Sylveniusgatan, Sweden). This ELISA employs a solid phase two-site sandwich enzyme immunoassay technique, in which two monoclonal antibodies are directed against separate antigens on the insulin molecule. Fifty uL of each serum sample in duplicates along with known concentrations of insulin standards were incubated with peroxidase-conjugated anti-insulin antibodies bound to microtitration wells. Unbound enzyme-labeled antibody was washed away. Bound conjugate were then detected by reaction with 3,3',5,5'-tetramethylbenzidine to give a colorimetric endpoint that was read spectrophotometrically at 450 nm. Absorbance values of known insulin standards were then plotted against insulin concentration on a cubic-spline scale.

Food Intake

Due to the potential of prepro-orexin and prepro-MCH levels to alter food intake we measured food intake during the period of the study where IUGR pups were consuming rodent chow. Pups were weaned at P21 and placed in individual cages. Regular mouse chow consumption was measured from P21 to P60. Of note, we could not measure food intake prior to weaning as the necessary handling of the pups would contribute to maternal rejection of the pups.

Statistics

Data were expressed as means \pm SEMs (standard error of the means). RT-PCR and ELISA data were analyzed with ANOVA with Fisher's protected least significance difference (PLSD) *post-hoc* test. Protein expression data were analyzed using the Mann-Whitney test. Statistical significance was declared at $p < 0.05$.

III. RESULTS

Hypothalamic Prepro-Orexin mRNA Levels

In IUGR and Sham Offspring at P60

Prepro-orexin mRNA levels were undetectable in IUGR pups at P7 (data not shown). Prepro-orexin mRNA levels also proved to be undetectable in sham pups at P7 (data not shown). IUGR did not alter prepro-orexin mRNA levels in males and females compared to sham males and females at P60 (Figure 1).

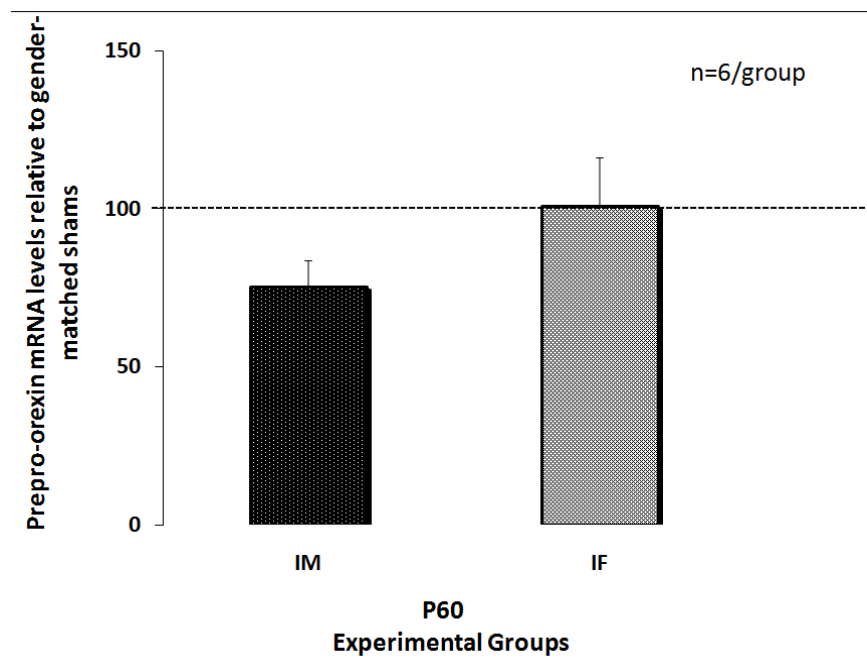


Figure 1: IUGR Prepro-orexin mRNA levels at P60

Hypothalamic Prepro-orexin Protein Levels In IUGR and
Sham Offspring At P7 and P60

Despite undetectable hypothalamic prepro-orexin mRNA levels, IUGR increased prepro-orexin protein levels in males as compared to sham males at P7 (Figure 2). IUGR, however, did not alter prepro-orexin protein levels in females as compared to sham females at P7. IUGR did not affect prepro-orexin protein levels in either males or females as compared to sham males and females at P60 (Figure 2).

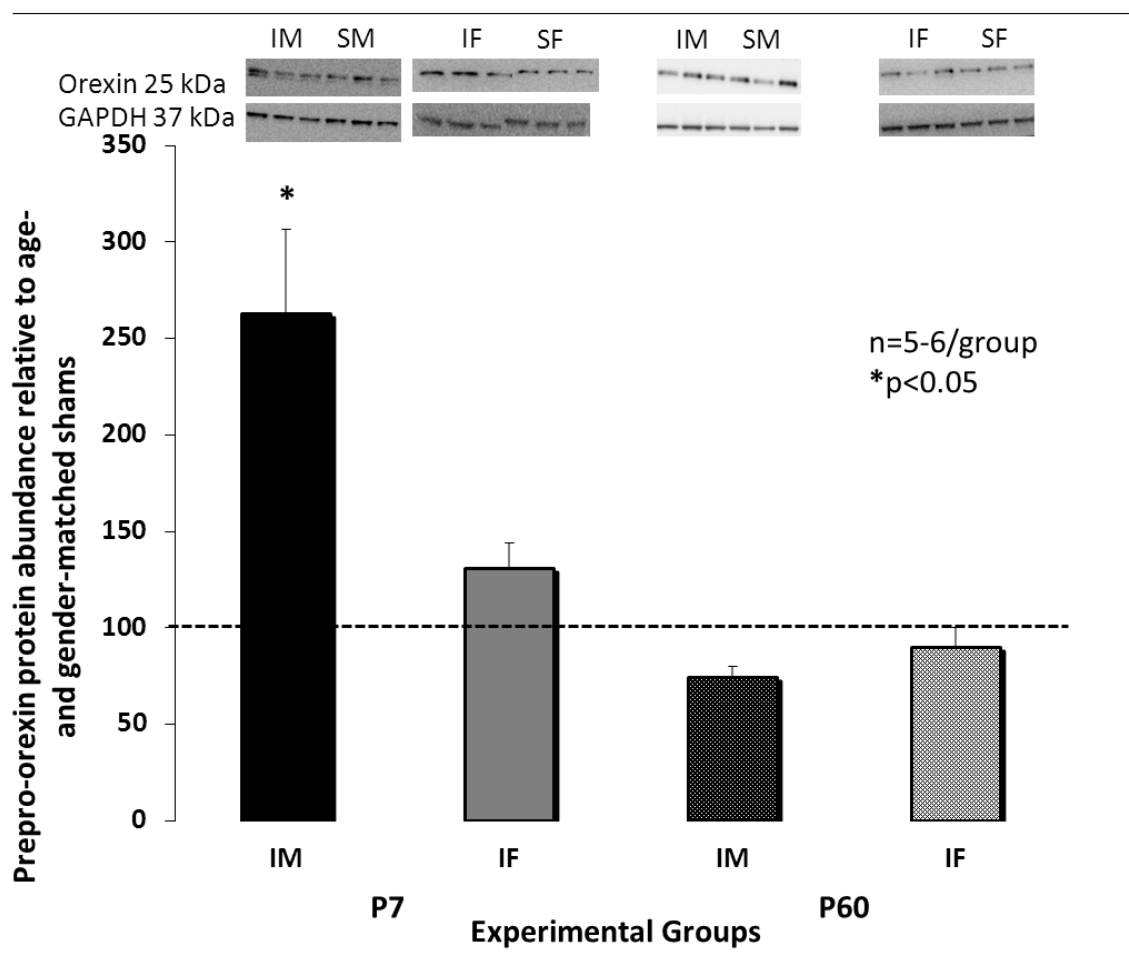


Figure 2: IUGR Prepro-orexin Protein Levels at P7 and P60

Hypothalamic Prepro-MCH mRNA Levels In IUGR and
Sham Offspring At P7 and P60

IUGR decreased prepro-MCH mRNA levels in males as compared to sham males at P7 (Figure 3). IUGR did not alter prepro-MCH mRNA levels in females as compared to sham females at P7. IUGR did not alter prepro-MCH mRNA levels in males and females as compared to sham males and females at P60 (Figure 3).

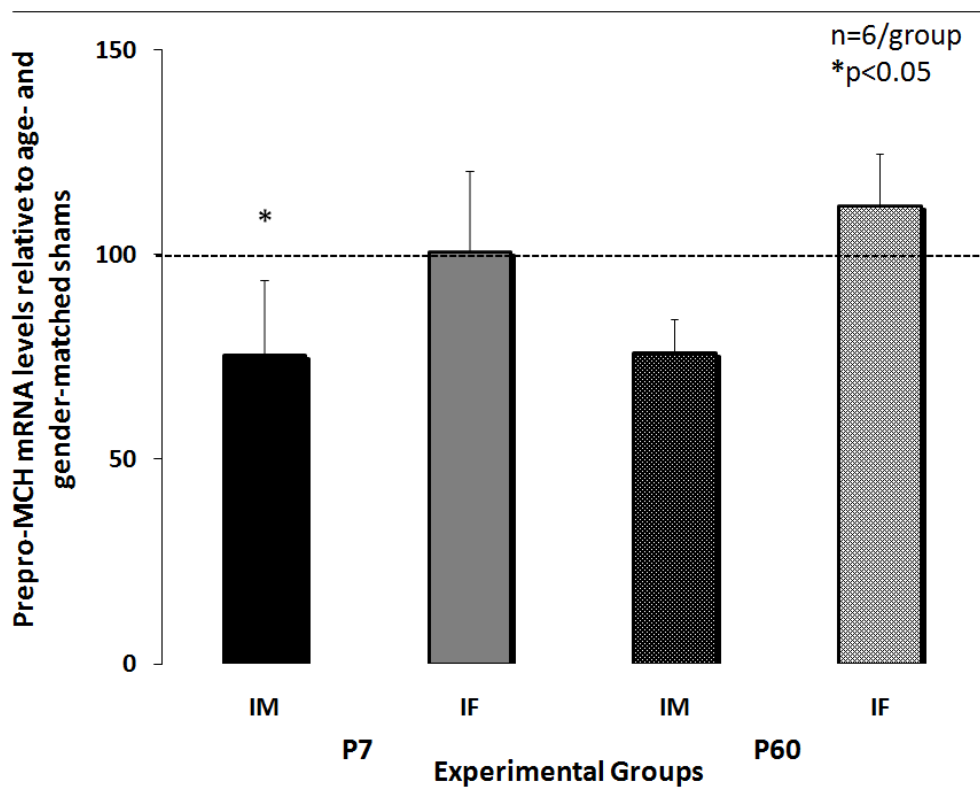


Figure 3: IUGR Prepro-MCH mRNA Levels at P7 and P60

Hypothalamic Prepro-MCH Protein Levels In IUGR and
Sham Offspring At P7 and P60

IUGR did not affect prepro-MCH protein levels in either males or females as compared to sham males and females at P7 (Figure 4). IUGR continued to have no effect on prepro-MCH protein levels in males and females as compared to sham males and females at P60 (Figure 4).

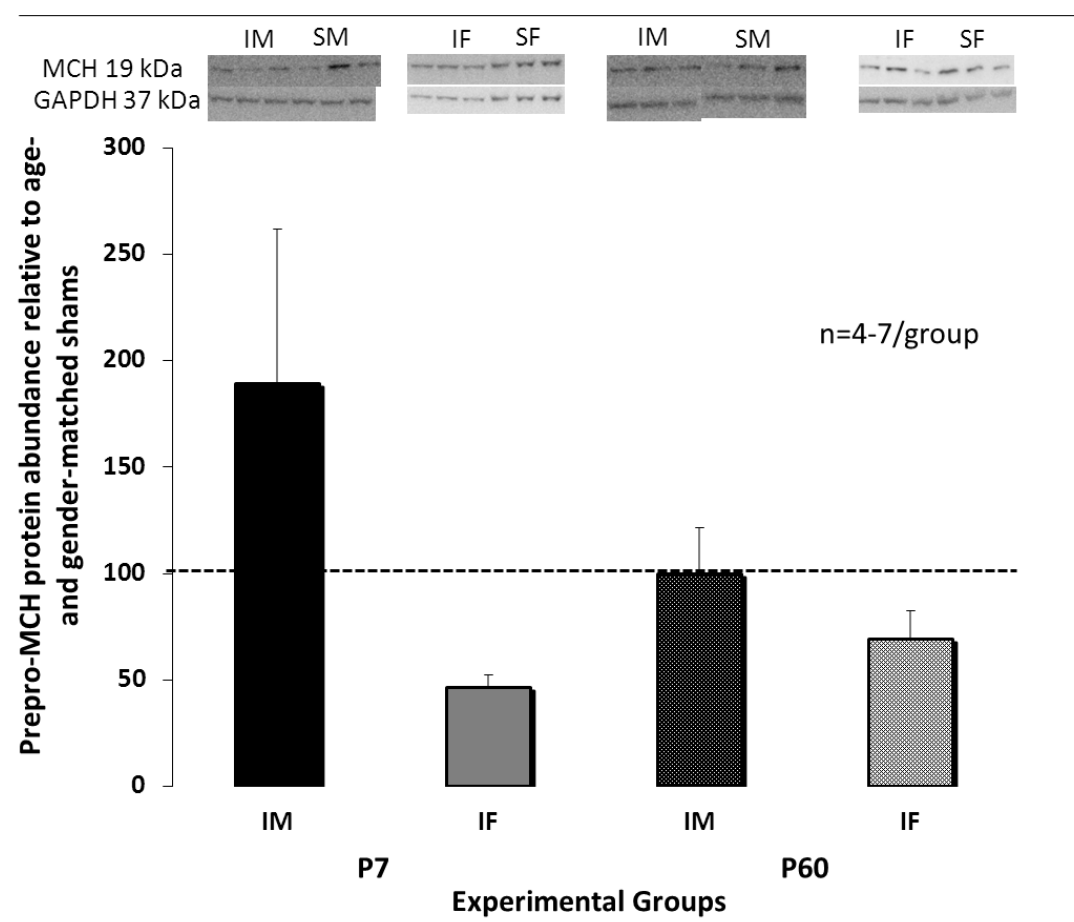


Figure 4: IUGR Prepro-MCH Protein Levels at P7 and P60

Serum Leptin Levels In IUGR and Sham

Offspring at P7 and P60

IUGR did not alter serum leptin levels at P7 relative to sham pups (Figure 5). IUGR continued to have no effect on serum leptin levels in males and females as compared to sham pups at P60 (Figure 5). However, serum leptin levels in IUGR males at P7 increased without significance [$p=0.08$].

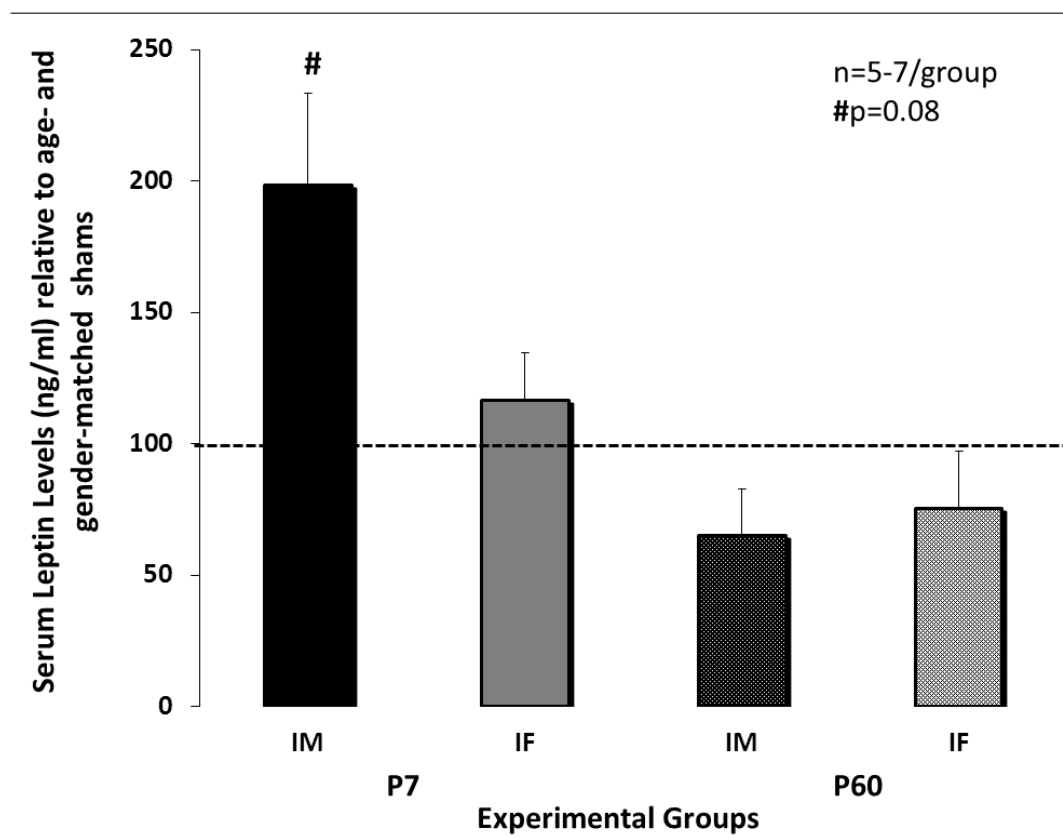


Figure 5: IUGR Serum Leptin Levels at P7 and P60

Serum Insulin Levels In IUGR and Sham

Offspring at P7 and P60

IUGR did not alter serum insulin levels in males and females as compared to sham pups at P7 in this IUGR mouse model (Figure 6). IUGR continued to have no effect in males and females as compared to sham offspring at P60 in this IUGR mouse model (Figure 6).

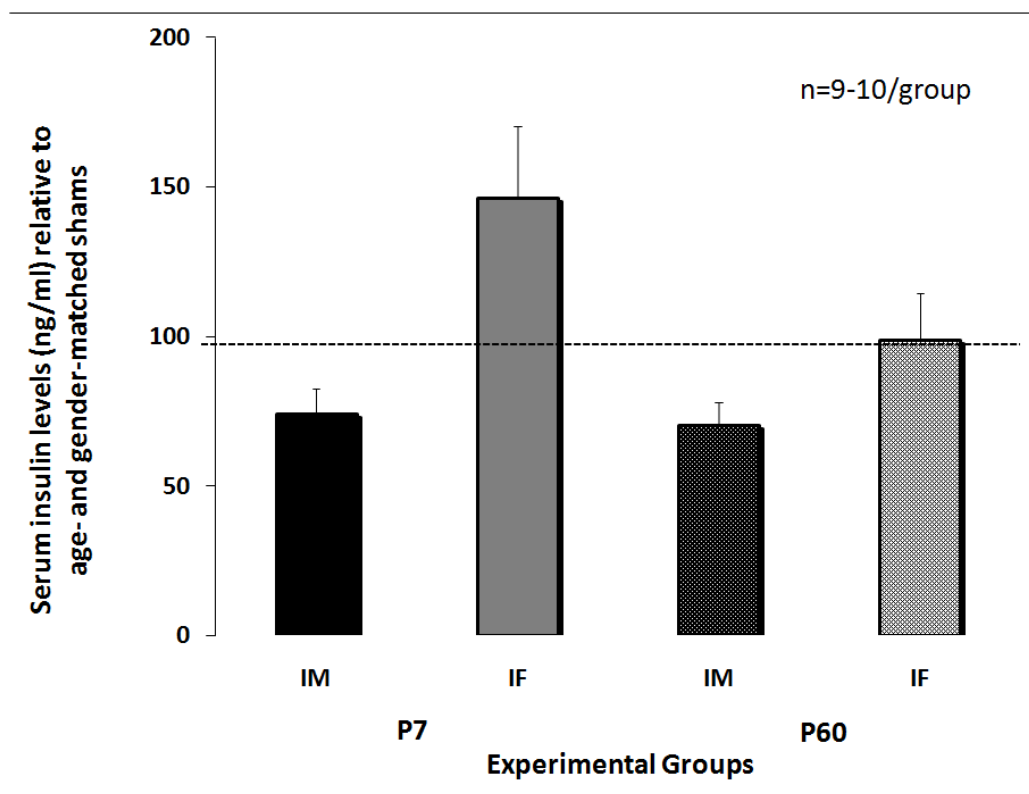


Figure 6: IUGR Serum Insulin Levels at P7 and P60

Food Intake In IUGR and Sham Offspring

From P21-P60

IUGR male offspring consumed similar amounts of food compared to sham male offspring respectively from P21-P60 (Figure 7). Furthermore, IUGR female pups as compared to sham female offspring also consumed similar amounts of food from P21-P60 (Figure 7).

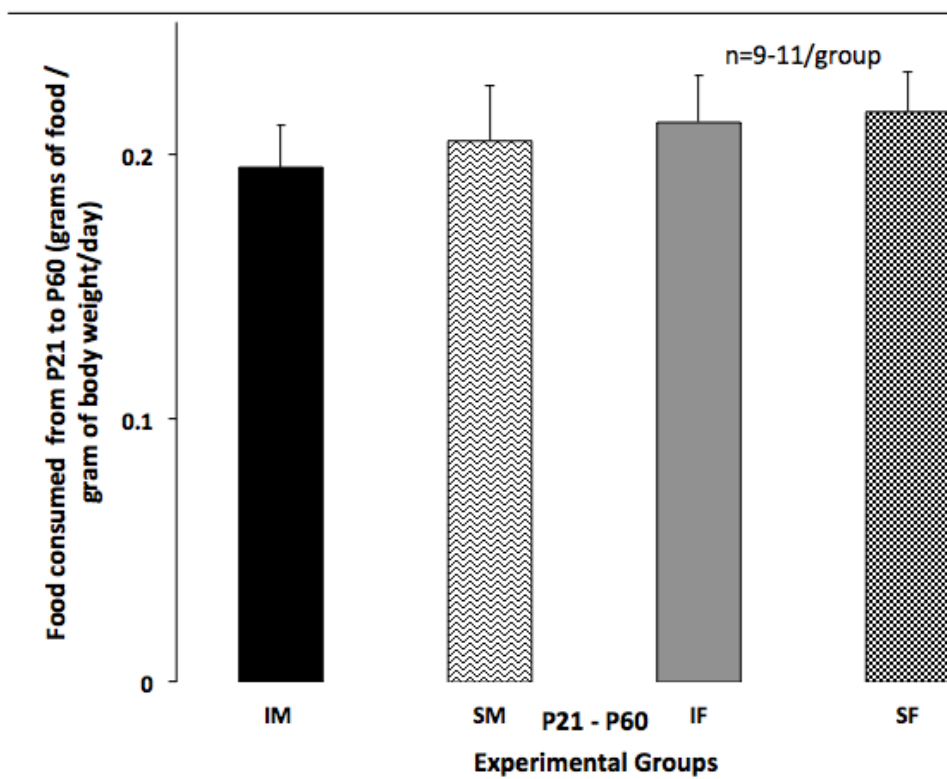


Figure 7: IUGR and Sham Offspring Food Intake from P21-P60

IV. DISCUSSION

The most significant finding of this project is that IUGR alters hypothalamic programming of prepro-orexin and prepro-MCH genes at P7 in IUGR male mice, potentially providing a mechanism for the rapid postnatal catch-up growth achieved in these IUGR males at P28. These findings agree with the current concept that IUGR can affect hypothalamic programming (3). However, to our knowledge, these findings are the first findings demonstrating that IUGR specifically affects the prepro-orexin- and prepro-MCH-producing neurons within the lateral hypothalamus in a gender-specific manner.

The increased prepro-orexin protein levels in IUGR male mice compared to sham male mice at P7 signify that IUGR has altered hypothalamic development leading to a maladjusted energy axis. Prepro-orexin levels are known to increase during a hypoglycemic state in rats (12). However, our P7 mouse pups are nursed by their cross-fostered dams up to the time of sacrifice, showing that despite having a normally fed state, prepro-orexin protein levels are still elevated over sham levels in IUGR males. Interestingly, IUGR females showed no such prepro-orexin protein increase compared to sham females at P7 despite being similarly nursed as IUGR males. The underlying mechanism behind this gender difference is unclear at this time. We are however intrigued by this gender disparity and speculate that altered testosterone to estradiol balance in the brain may be responsible for the dysregulation seen in IUGR males. We know from another IUGR model that IUGR males have increased serum testosterone

levels and decreased aromatase expression in the hippocampus of the brain (21).

Aromatase is the enzyme responsible for the conversion of testosterone to its metabolite estradiol. The exposure of the developing hypothalamus to an improper testosterone:estradiol ratio may hinder its proper development to result in altered hypothalamic programming.

The decrease in prepro-MCH mRNA levels in P7 IUGR male mice as compared to sham mice provides another piece of evidence that IUGR alters hypothalamic programming in our model. The discrepancy between prepro-MCH mRNA and protein levels could reflect altered mRNA or protein stability in P7 IUGR males. While the mechanism behind this finding requires further investigation, it further demonstrates IUGR induced hypothalamic programming in IUGR males.

The extra-hypothalamic leptin surge is essential to placement and further development of leptin receptors and neuronal growth (3). If this leptin surge occurs prematurely, hypothalamic organization is altered (3,18). In our model, IUGR males exhibited a trend of increased serum leptin levels at P7, and while the increase did not reach statistical significance, the increase is likely biologically significant. Reinvestigation with an increased sample size may demonstrate significance. A premature leptin surge in mice before P14 is known to decrease the anorexigenic gene expression of proopiomelanocortin (POMC), thereby establishing dysfunctional programming and signaling in the hypothalamus thereby producing a phenotype prone to rapid, catch-up growth (3,18), such as that observed in our model. IUGR did not elicit changes in hypothalamic prepro-orexin and prepro-MCH mRNA or protein levels, serum leptin and insulin levels, and food intake in either gender at P60. The interpretation here

is different from P7 because these genes at P60 have a greater role in governing food intake than hypothalamic development; therefore these results show that food intake is not the perturbed mechanism for increased catch-growth in IUGR males.

We recognize that certain limitations exist in our study. We have examined prepro-orexin and prepro-MCH mRNA and protein levels; however, to further elucidate the effects of these ligands, we need to investigate orexin and MCH receptors as well as the downstream targets of those receptors to provide a more complete picture of the orexin and MCH system in these IUGR pups. Furthermore, milk intake could not be measured from P0-P21 thereby limiting information on actual caloric intake of the IUGR male pups as compared to sham male pups during this critical time of rapid catch-up growth. Additionally, hypothalamic development time points differ between mice and humans. While similar programming may occur in both species, all of human hypothalamic development occurs *in utero* while murine hypothalamic development spans the end of gestation into the first weeks of postnatal life, therefore data cannot be extrapolated directly to humans (3). The major benefit of using an animal model, however, is the allowance for exploration of mechanisms of disease in a compressed developmental time span which would otherwise take years to achieve in humans.

In summary, IUGR's alterations to hypothalamic programming contribute to a phenotype destined for obesity and its co-morbidities (3). Limited knowledge currently exists regarding hypothalamic programming and development in humans, giving rise to the importance of examining all alterations to hypothalamic changes in an animal model that occur as a result of IUGR. Only by understanding the mechanisms behind IUGR-induced obesity can we begin to institute therapy targeted at those aberrancies.

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